Multiple Residues Specify External Tetraethylammonium Blockade in Voltage-Gated Potassium Channels

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ABSTRACT We have mapped residues in the carboxyl half of the P region of a voltage-gated K^+ channel that influence external tetraethylammonium (TEA) block. Fifteen amino acids were substituted with cysteine and expressed in oocytes from monomeric or heterodimeric cRNAs. From a total of six mutant channels with altered TEA sensitivity, three were susceptible to modification by extracellularly applied charged methanethiosulfonates (MTSX). Another residue did not affect TEA block but was protected from MTSX by TEA. MTSX modification of position Y380C, thought to form the TEA binding site, affected TEA affinity only moderately, and this effect could be reversed by additional charge transfer from an oppositely charged MTSX analog. The results show that TEA block is modulated from multiple sites, including residues located deep in the pore and that several side chains besides that of Y380 are exposed at the TEA receptor.

INTRODUCTION

Tetraethylammonium (TEA) is widely used as a probe for the external mouth of voltage-dependent K^+ channels (Stanfield, 1983). A single TEA ion binds to the permeation pathway for a brief time, blocking ion flow (Hille, 1967). Conversely, multiple ion occupancy of the pore may destabilize bound TEA ions (Armstrong, 1971; Newland et al., 1992). A third partner in the interaction between TEA and permeant ions inside the channel is contributed by the amino acidic lining of the pore (MacKinnon and Yellen, 1990; Kavanaugh at al., 1991, 1992; Yellen et al., 1991; Heginbotham and MacKinnon, 1992). Steric, electrostatic, or hydrophobic effects have been proposed to explain the influence of several residues on the binding of TEA and other K^+ pore blockers (MacKinnon and Yellen, 1990; Heginbotham and MacKinnon, 1992; Shieh and Kirsch, 1994). Therefore, identification of the side chains that affect TEA block is a prerequisite for the mechanistic analysis of ion permeation and blockade.

Previous work identified determinants of TEA block in the S5-S6 linker domain (including the P region) of voltage-gated K^+ channels (Yellen et al., 1991; Yool and Schwarz, 1991; Hartmann et al., 1991). In *Shaker* channels, position D431 favors TEA binding via through-space electrostatic interactions (MacKinnon and Yellen, 1990), whereas substitutions T449Y and T449F enhanced TEA block (Heginbotham and MacKinnon, 1992). In a Kv2.1/Kv3.1 chimeric channel (Hartmann et al., 1991), K382, thought to lie near the external channel mouth, modulated external TEA block and current-voltage rectification properties in a manner consistent with an electrostatic effect (Kirsch et al., 1992). In contrast, I369 and V374, considered to be "deep pore" residues (Kirsch et al., 1992), also modulated external TEA block, possibly by affecting ion occupancy in the pore. Thus, TEA binding appears to be influenced from several sites. The purpose of our investigations was 1) to identify positions that determine TEA blockade, and 2) to establish which of these residues are likely to be exposed at the surface where TEA binds. To identify side chains that affect TEA block in Kv2.1 we substituted each of 15 amino acids in the carboxyl half of the P region with cysteine and assayed the TEA sensitivity of the currents expressed by the mutants under voltage clamp. We chose cysteine 1) because it is a small, intermediate polarity residue (Creighton, 1993) and 2) because its side chain can be selectively and covalently modified from aqueous phase by three charged, hydrophilic methanethiosulfonate analogues (methanethiosulfonate-ethylammonium, MTSEA; -ethyltrimethylammonium, MTSET; and -ethylsulfonate, MTSES; Akabas et al., 1992, 1994). Although different in shape, MTSEA and MTSET are positively charged and comparable in size to TEA (approximate volumes are: MTSEA, 153 $Å^3$; MTSET, 195 $Å^3$; and TEA, 130 $Å^3$), providing us with a set of similar volume probes.

We found six scattered substitutions that affected TEA block (at positions T370, T373, Y376, D378, Y380, and K382 (see Fig. 1). Among these, only three positions (D378, Y380 and K382) were also accessible to MTSX probes. Position I379, a residue that did not influence TEA sensitivity when substituted to cysteine, was available to MTSX derivatives and could be protected from MTSET by simultaneous TEA application. Our results suggest that the sensitivity of the pore to TEA block is modulated from multiple sites, some of which, because of surface exposure at the external mouth, are more likely to act through direct interactions at the receptor site. Preliminary accounts of these

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results have been previously reported (Pascual et al., 1994, 1995).

MATERIALS AND METHODS

Mutagenesis and dimer construction

Recombinant DNA techniques have been previously described in Kirsch et al. (1995). Briefly, we synthesized mutant monomer cDNAs by ligation of polymerase chain reaction fragments including convenient restriction endonuclease sites and point mutations into host Kv2.1 constructs carrying a 351-amino acid carboxyl terminal deletion. All macroscopic and single-channel properties of this deletion mutant were indistinguishable from full-length wild type (WT) channels. All polymerase chain reaction-derived segments and ligation points were fully sequenced. For dimer (referred to as WT-WT, K382C-WT, etc.) construction, we transferred part (1.2 kb) of the coding region of mutant monomers to dimeric Kv2.1 cDNAs from which a complementary fragment was previously excised. Ligation points and mutant areas of intact dimers were again sequenced without preliminary manipulation by identifying mismatching sequence bands against a consensus background originated from both dimer repeats. cRNAs were synthesized, stored, and injected into *Xenopus laevis* oocytes as described in Drewe et al. (1994). Further verification of cRNA integrity was provided by denaturing formaldehyde-agarose gel electrophoresis using RNA size standards.

Whole-cell recording

Two-microelectrode voltage clamp recording was performed as described in Kirsch et al. (1995). Bathing solution consisted of (in mM): 60 NaOH, 51 KOH, 2 $CaCl_2$, 120 methanesulfonic acid, 11.5 *N*-methyl-D-glucamine, 10 HEPES, pH 7.35. Methanethiosulfonates were dissolved in this solution immediately before application to the oocyte. All experiments were performed at room temperature (20–23°C). Data are expressed as means ± SEM where appropriate.

Preliminary functional stoichiometry tests for the dimer constructs have been described in Kirsch et al. (1995). We took advantage of the TEA IC_{50} values specified by Y380C and K382C to further test the hypothesis that dimer subunits assembled as expected (two dimers generating one conducting pore). To address whether dimer subunits were incorporated into a single population of channels, we determined the TEA dose-response profile for K382C-WT and Y380C-WT channels. Both curves were intermediate between those obtained for the corresponding monomeric mutant and WT and could be accurately fitted by single 1:1 binding isotherms (see Fig. 5 and Table 1). Therefore, it appears that these dimers originated phenotypically uniform channels. To test whether the observed changes in TEA sensitivity derived from the manipulations performed at the amino and carboxyl channel termini, we assessed the TEA affinity of K382C-K382C dimers. As expected, we obtained similar IC_{50} values when comparing K382C-K382C dimers with K382C monomers (see Fig. 5 and Table 1). Finally, we asked whether subunit position influenced TEA sensitivity by expressing K382C-WT and WT-K382C dimers. Both constructs displayed identical IC_{50} values, suggesting that both repeats participate in pore assembly, regardless of the order in which they are linked. Nevertheless, the experiments described here do not provide stoichiometric evidence in cases in which a mutant dimer behaves indistinguishably from WT-WT dimers. Substitutions G375C-WT, G377C-WT, and P381C-WT generated channels that resembled WT-WT in terms of macroscopic gating, TEA block, and MTSX insensitivity. Therefore, we cannot rule out preferential subunit assembly in these cases. To test whether the second repeat of a dimer could generate functional channels by self-assembly, we introduced the deletion Y376-G377-D378 in the first repeat of a dimer. This deletion rendered dimer channels nonfunctional, as previously observed with its monomeric form (Pascual et al., 1995b). To rule out the participation of spurious translation initiation sites downstream from the first cRNA repeat, we generated 1) a tetrameric clone by the insertion of additional cDNA repeats and 2) an analogous tetramer in which nucleotide 1024 (located in a triplet encoding a residue in the P region of the first repeat) was deleted, shifting the rest of the first and the last three repeats out of frame. Pore properties of channels generated by the tetrameric construct were identical to those observed in WT channels, whereas the frame-shifted tetramer cRNA failed to produce voltage-dependent or leak currents (n = 5), suggesting that cRNA repeats downstream from the first one lack the ability to be translated separately.

RESULTS AND DISCUSSION

Residues affecting TEA block

From our survey of 15 amino acids (Fig. 1), seven substitutions (at positions T370, T373, G375, Y376, G377, D378, and P381) failed to produce whole-cell currents with sufficient amplitude (>1 μA) for detailed characterization. Significantly, all these mutations involved residues conserved in voltage-dependent K^+ channels across evolution. We overcame this problem by genetic manipulation; all substitutions that did not produce functional channels were transferred to Kv2.1 dimer constructs containing one WT repeat as previously described (Kirsch et al., 1995). This procedure provided us with a rescue system that generated hybrid tandem subunits that presumably assembled pairwise to form functional channels (see Materials and Methods).

Whole-cell currents from oocytes injected with nine monomeric or dimeric substitutions (I369C, M371C-WT, T372C-WT, V374C, G375C-WT, G377C-WT, I379C, P381C-WT, and T383C) were blocked by TEA with IC_{50} values similar to those observed in cells expressing WT and WT-WT channels (Fig. 2 and Table 1). Although M371C and T372C-injected oocytes expressed large currents, we tested the dimer forms of M371C and T372C because TEA slowed a fast inactivation process introduced by the monomeric mutations that interfered with steady state block measurements. The inactivation of M371C could be fitted using an exponential function with time constant 0.6 s at 40 mV, in contrast with the time constant for WT, which was 3.3 s. T372C channels inactivated at intermediate rates between the previous two values. M371C-WT and T372C-WT

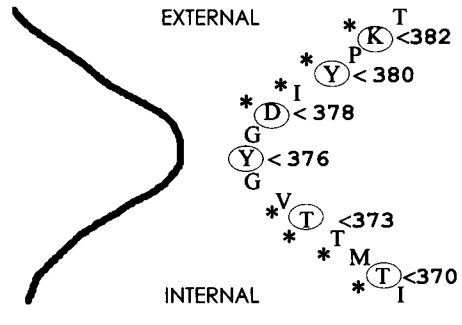

FIGURE 1 Putative transmembrane topology for residues 369–383 in the P region of Kv2.1. A cross-sectional, hourglass-shaped scheme of the narrower part of the pore is represented. Circled: residues that affected external TEA block (causing a >2-fold change in the IC_{50} value) when replaced with cysteine. Asterisks indicate cysteine substitutions available to extracellularly or intracellularly applied MTSET (Pascual et al., 1995b).

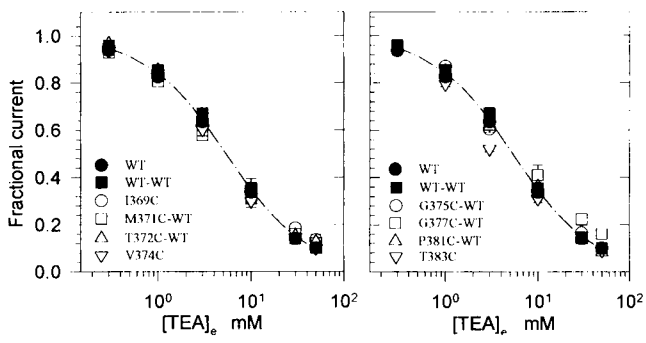

FIGURE 2 TEA dose-response measurements in oocytes expressing Kv2.1 monomers (WT), dimers (WT-WT), and mutant constructs. Steady-state block of outward currents by TEA was measured at the end of 450-ms test pulses to 40 mV. Each point represents average ± SEM measurements from 3 to 5 oocytes. The dashed-dotted line corresponds to a 1:1 binding curve fitted to WT and WT-WT data with a Hill coefficient of 1.

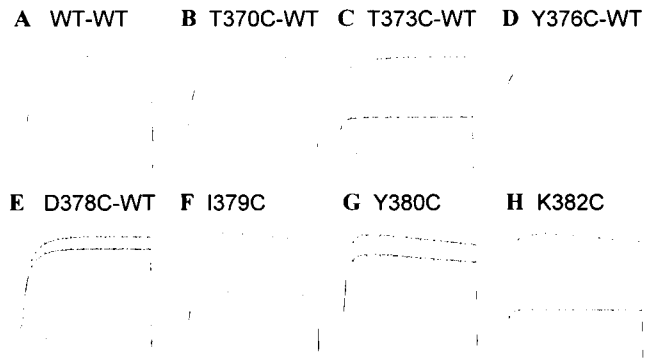

FIGURE 3 (A- H) Block of dimer (WT-WT) Kv2.1 and mutant channels by 10 mM TEA. Cells were depolarized to 40 mV. Vertical calibration bars = 2 μA; horizontal calibration bars = 150 ms.

dimers displayed slower inactivation kinetics that were insensitive to TEA, allowing for accurate TEA dose-response computations. The TEA IC_{50} values for both M371C-WT and T372C-WT channels were similar to WT. The remaining six mutations (T370C-WT, Y376C-WT, D378C-WT, Y380C, and K382C) significantly altered TEA IC_{50} values, while the substitution T373C-WT had a more modest effect (Figs. 3 and 4 and Table 1). As expected from previous work, substitution K382C in the monomer construct, that completely removes positive charge from the external mouth of the pore, enhanced TEA affinity (Kirsch et al., 1992). Partial neutralization (in the heterodimer) was less effective. By contrast, Y380C, which removes an aromatic side chain, decreased TEA sensitivity (Fig. 4 and Table 1; Heginbotham and MacKinnon, 1992; Kavanaugh et al., 1991). In addition to K382C and Y380C expressed as monomer constructs, channels expressed as dimer constructs carrying cysteine at positions 370, 376, and 378 also showed marked changes in TEA sensitivity (Fig. 4 and Table 1). Surprisingly, these sites include residues (370 and 376) thought to lie deeper in the pore, in a TEA-inaccessible region (Heginbotham et al., 1992). In fact, the side chains of T370, T372, T373, and V374 line the opposite (intracellular) mouth of the channel (Pascual et al., 1995b). These results can be rationalized by considering that ion occupancy of the channel modulates the affinity of the TEA receptors at the pore mouths (Newland et al., 1992). At several positions we made substitutions other than cysteine that were designed to be more conservative and potentially less disruptive of pore structure. As shown in Table 1, for position T370 a cysteine substitution drastically altered external TEA block, whereas the conservative serine substitution had no effect. We suggest that the effect of cysteine substitution may be to alter pore structure and/or ion occupancy. Position Y376 may fall in the same category. Unfortunately, we have only attempted another substitution at Y376 with the aromatic tryptophan which resulted in a drastic reduction in the level of current when expressed as a heterodimer. By contrast, at position D378 a more conservative (in terms of hydrophyllicity and volume) replacement with threonine was more effective than cysteine in preventing TEA block. This position (and Y380, as shown by Heginbotham and MacKinnon, 1992), may have a direct influence on TEA binding (Kirsch et al., 1995). As discussed below, the external surface exposure of these residues and the protection of an additional one (I379C) by TEA provide additional support for this hypothesis.

TABLE 1 External TEA sensitivity in Kv2.1 mutants

Monomer clone	TEA IC_{50} (mM)	n	Dimer clone	TEA IC_{50} (mM)	n
WT	5.4 ± 0.2	4	WT-WT	5.7 ± 0.2	4
I369C	5.8 ± 0.5	4	T370C-WT	31.0 ± 0.2	4
V374C	4.8 ± 0.3	4	T370S-WT	4.8 ± 0.9	4
I379C	4.2 ± 0.5	4	M371C-WT	4.4 ± 0.3	3
Y380C	34.5 ± 0.2	9	T372C-WT	5.5 ± 0.2	3
K382C	1.4 ± 0.1	4	T373C-WT	2.5 ± 0.1	4
T383C	3.9 ± 0.3	5	T373S-WT	2.9 ± 0.1	3
			T373V-WT	3.0 ± 0.7	4
			G375C-WT	5.2 ± 0.4	4
			G375A-WT	9.6 ± 1.2	5
			Y376C-WT	96.5 ± 0.2	5
			G377C-WT	6.7 ± 0.4	4
			G337A-WT	8.6 ± 1.3	4
			D378C-WT	55.0 ± 0.3	6
			D378T-WT	1305 ± 220*	4
			Y380C-WT	14.2 ± 0.4	5
			Y380S-WT	13.9 ± 4.2	3
			P381C-WT	5.2 ± 0.3	5
			K382C-WT	3.2 ± 0.2	5
			WT-K382C	3.2 ± 0.2	3
			K382C-K382C	1.7 ± 0.1	3

Dose-response relationships were obtained under whole-cell clamp by measuring steady-state outward current at the end of 450-ms test pulses to 40 mV in the presence of five to seven TEA concentrations The data points were fitted using single 1:1 binding isotherms of the form: Fractional current = $(I_{TEA}/I_{CONTROL}) \times 100$. IC_{50} mean derivations from WT and WT-WT greater than twofold are underlined.

* Extrapolation from reversible block at 120 mM TEA (Kirsch et al., 1995).

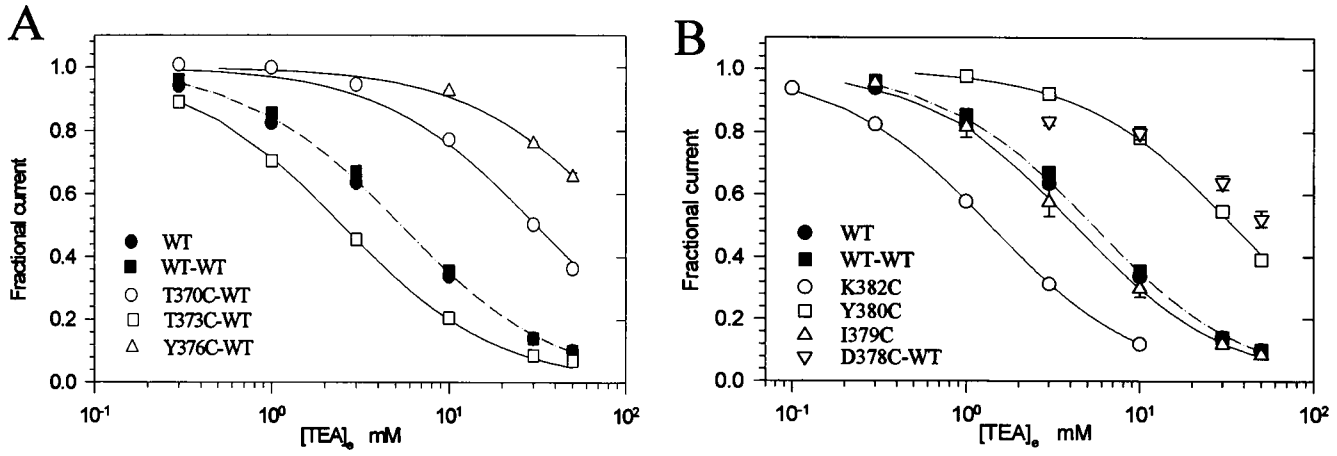

FIGURE 4 TEA block in MTSX-inaccessible and (*A*) MTSX-available cysteine substitutions (*B*). Data were obtained from 5 to 9 oocytes for each mutant. The reference dashed-dotted line, replotted from Fig. 2, represents fitted WT sensitivity.

Residues lining the external channel mouth

We took advantage of the size and charge similarity between TEA and positively charged, channel and membrane impermeant MTSX probes to identify residues that line the TEA binding area. We assumed that MTSEA and MTSET may visit TEA-interacting sites when they access the pore. However, as opposed to the binding of TEA, reaction of MTSX with cysteinyl sulfhydryls forms a disulfide bond that cannot be broken by ion occupancy of the pore. This approach would help distinguish between non-reactive, "deep-pore" positions and MTSX-available residues located in the external channel surface. In control experiments (Pascual et al., 1995b) extracellularly applied MTSX analogues did not affect the macroscopic or unitary conduction and kinetic properties of WT and WT-WT channels.

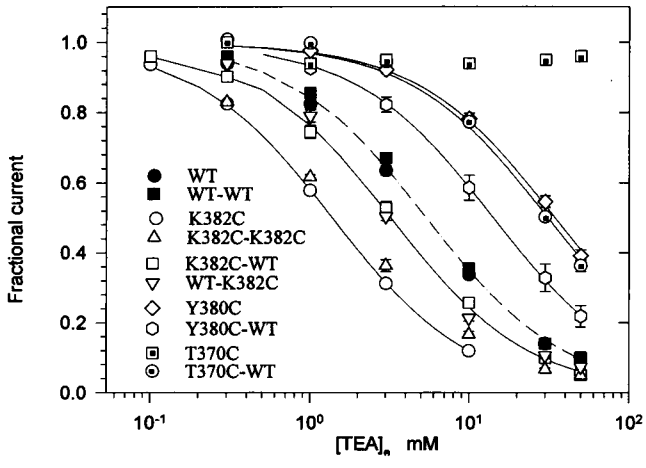

FIGURE 5 TEA block in monomer and related dimer constructs. Curve lines are fitted 1:1 binding isotherms with Hill coefficient 1. Each point was obtained from 3 to 9 oocyte determinations, except for T370C, which represents the only cell from which currents greater than 1 μA could be recorded.

Of the six positions that affected TEA block, only D378C-WT, Y380C, and K382C channels apparently reacted with MTSX, displaying different degrees of current modification after saturating applications (Table 2). Therefore, these positions may be exposed in the neighborhood of the external channel mouth. We predicted that MTSX modification of superficially located residues should affect TEA sensitivity. Indeed, TEA IC_{50} values shifted after MTSX modification of Y380C and K382C channels (Fig. 6 and Table 3). MTSEA and MTSET application reduced TEA block of Y380C and K382C channels, while modification by MTSES had the opposite effect (Table 3). In contrast, the IC_{50} of D378C-WT, already drastically increased over WT-WT, was not altered any further by MTSX treatment, even though D378C-WT currents were significantly inhibited by MTSX (Table 3). This unexpected result suggests that residual TEA block after mutation no longer involves position 378. If so the structural changes induced by substitutions at this locus may be extensive. Indeed, a more conservative substitution, D378T-WT, causes drastic changes in ion conduction and TEA block, as well as more subtle changes in Na^+/K^+ selectivity (Kirsch et al., 1995).

I379C is exposed near the TEA receptor

Interestingly, I379C, a mutation that did not affect TEA sensitivity (TEA IC_{50} = 4.2 mM; Table 1), was available

TABLE 2 Outward current modification by MTSX

Clone	MTSEA	*n*	MTSET	*n*	MTSES	*n*
K382C	78 ± 3	3	72 ± 1	3	105 ± 6	3
Y380C	82 ± 2	17	44 ± 5	20	137 ± 3	14
I379C	32 ± 3	3	5 ± 2	3	25 ± 2	3
D378C-WT	63 ± 4	3	50 ± 3	3	71 ± 5	4

MTSX analogs were applied to saturation and the change in outward current at 50 mV was measured considering unmodified current levels = 100. Values indicate percent current.

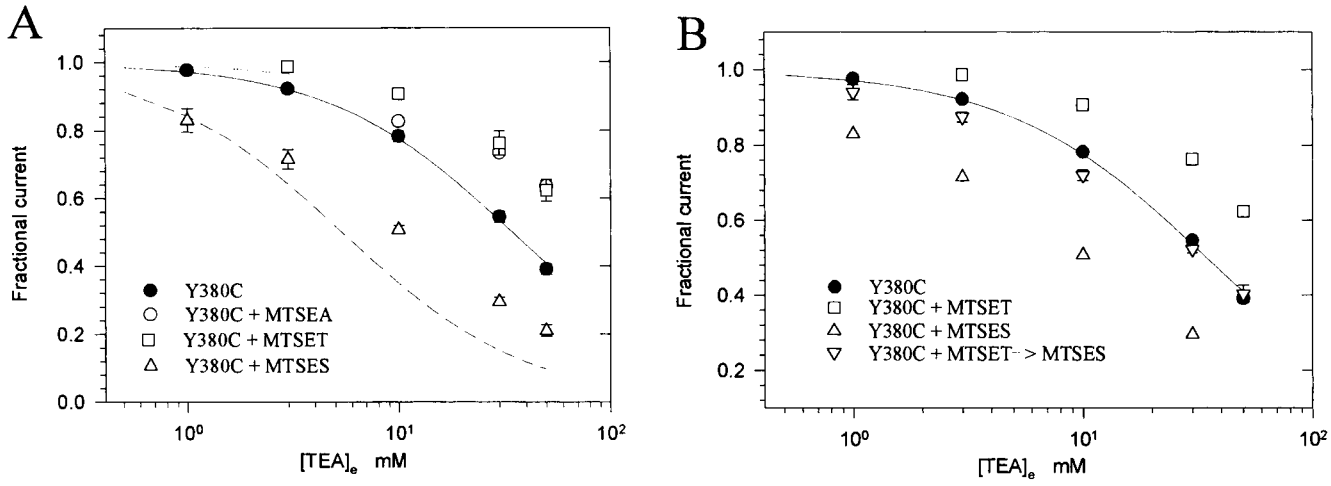

FIGURE 6 TEA block of Y380C channels before and after MTSX application. (*A*) Solid symbols were obtained from 9 Y380C cells, whereas empty symbols represent measurements from 3 to 5 oocytes saturated after MTSX application. Dotted lines correspond to fitted binding isotherms with Hill coefficient 1. For MTSES-modified Y380C channels, a Hill coefficient of 0.8 gave best fits. For reference, the WT dose-response curve is replotted from Fig. 2 using a dashed-dotted line. (*B*) TEA block in Y380C channels sequentially modified by MTSET and MTSES. The cells were exposed to saturating applications of MTSET and then perfused with MTSES to new saturation. Data obtained from three experiments. For reference, the data points and fitted binding isotherms of unmodified, MTSET modified, and MTSES modified Y380C channels are replotted from *A*.

for MTSX modification (Table 2 and Kürz et al., 1995). MTSEA and MTSES application to I379C channels reduced TEA block (Table 3). Additionally, TEA occupancy partially protected I379C channels from MTSET modification (Fig. 7). Although we support the view that the chemical nature of the side chain present at position 379 (whether I, M, or C) does not influence TEA affinity (Kirsch et al., 1992), we propose that I379C is exposed in the vicinity of, or deeper past the TEA binding area, such that bound TEA prevents or slows access of MTSET to its side chain.

In contrast with these observations, simultaneous MTSET and TEA application to the other MTSX-susceptible mutants (D378C-WT, Y380C, K382C) did not affect the time course of current reduction compared to similar experiments in which MTSET was applied alone (Table 3). The fact that TEA (at 50 mM) offered no protection from MTSET in D378C-WT (TEA IC_{50} = 55 mM) and Y380C (TEA IC_{50} = 35 mM) channels may be due to insufficient TEA occupancy of the mutant pores, as deduced from their higher IC_{50} values (Table 1), or to the independence between the TEA and MTSX binding sites. Therefore, we could not evaluate the negative protection results at D378C-WT and Y380C. Nevertheless, K382C channels, which were slightly more sensitive to TEA than I379C channels (TEA IC_{50}'s = 1.4 mM vs. 4.2 mM, respectively; Table 1) were not protected from MTSET under identical conditions. Therefore, partial occupancy of K382C channels by TEA was compatible with an MTSX-accessible side chain at that position. This result can be rationalized by assuming that K382 lies at a more remote location from the TEA binding area and that it affects TEA block by through-space electrostatic interactions (Kirsch et al., 1992) .

TEA block of Y380C modified channels

We were surprised, however, that the transfer of a MTSET head group to Y380C, proposed to lie at the TEA binding site (Heginbotham and MacKinnon, 1992), did not completely block currents (44% reduction at saturating concentrations and 50 mV; Table 2) and reduced TEA affinity only threefold (IC_{50} increased from 35 mM to 89 mM, Table 3). We reasoned that if the TEA binding site were formed by a cage of four phenolic rings at position 380, disruption of the cage by cysteine replacement should render the mutant channels much less sensitive to TEA. Additionally, modi-

TABLE 3 TEA IC_{50} (in mM) in mutant channels after MTSX application

	D378C-WT	*n*	I379C	*n*	Y380C	*n*	K382C	*n*
MTSEA	58.3 ± 0.5	3	19.7 ± 0.9	3	80.1 ± 0.5	3	4.8 ± 0.3	4
MTSET	54.3 ± 2.2	3	N.D.		89.1 ± 0.1	9	3.9 ± 0.1	4
MTSES	55.1 ± 1.3	3	38.1 ± 1.0	3	9.9 ± 1.1	4	0.8 ± 0.1	4
MTSET → MTSES	N.D.		N.D.		30.1 ± 0.3	4	N.D.	
TEA protection	no		yes		no		no	

Bottom row indicates channels in which TEA (50 mM) partially protected from MTSET modification. Small residual currents in oocytes expressing I379C channels after MTSET treatment prevented estimation of their sensitivity to TEA (N.D.).

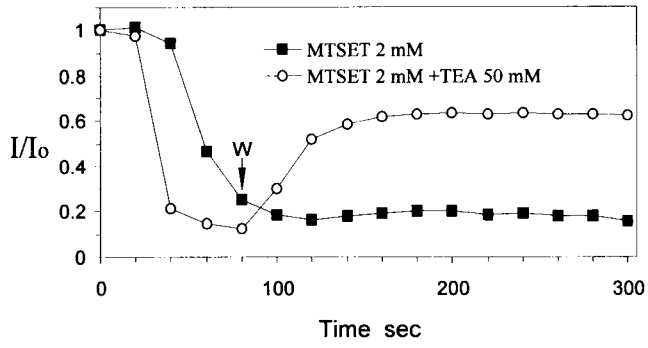

FIGURE 7 Time course of current changes in TEA and MTSET-treated I379C channels. At time 0, 2 mM MTSET was applied either alone (■) or in combination with 50 mM TEA (○). After 80 s the chamber was perfused with drug-free saline. Currents amplitudes were measured at the end of 450-ms test pulses to 40 mV. Currents were normalized using the expression I/I_o, where I_o represents maximum (control) current and I is the current measured during modification. The results shown were obtained from one of three similar experiments.

fication of Y380C channels with MTSET, comparable in size to TEA, should abolish any residual TEA sensitivity. Contrary to expectation, Y380C channels displayed only a moderate (sixfold) shift in TEA sensitivity compared to WT channels (IC_{50}'s = 35 vs. 5.3 mM, respectively, Table 1), and MTSET reduced IC_{50} values by an additional 2.5-fold factor (Table 3). Furthermore, MTSES application to MTSET-saturated Y380C channels restored IC_{50} values to unmodified levels (Fig. 6 and Table 3). Analogously, instantaneous current-voltage relationships in Y380C MTSET-modified channels reverted to unmodified levels after MTSES application (not shown). None of these effects were observed after application of MTSES to MTSEA- or MTSET-modified D378C-WT, I379C and K382C channels, which were insensitive to MTSES after one saturating MTSEA or MTSET application. All of these results were independent of the order in which the compounds were used. Therefore, it appears that more than one side chain (of a total of four) can be modified at position Y380C, provided that opposite-charge reagents are applied. Our results at position Y380C suggest that the presence of at least two bound, opposite-charge MTSX derivatives interferes minimally with TEA block and ion conduction, despite the reduction in pore diameter caused by the probes.

Therefore, it seems unlikely that Y380 is the main determinant for TEA binding in Kv2.1 channels. Although important for specifying differences in TEA sensitivity among K channels (Kavanaugh et al., 1991; Heginbotham and MacKinnon, 1992), we found that residues (at least four) other than 380 influence TEA block. Among these, K382 and D378 are exposed in the vicinity of the external channel mouth, and an additional side chain (I379) lies near the TEA binding area. In contrast, the remaining positions line deeper parts of the pore (Kirsch et al., 1992; Pascual et al., 1995b). Our results support the view that the affinity of the channel mouths for selective blockers can be modulated from multiple sites, some of which are more likely to act through indirect interactions from remote positions (T370, T373, Y376, K382) and others that act directly (D378 and Y380).

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